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## Detection and evaluation of antibiotic-resistant *Escherichia coli* strains in birds of prey from the Kerman Province, Iran



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### ABSTRACT

Raptors play an important role in the ecosystem as top predators. They can transmit diseases over long distances, affecting the environment, humans, and animals, in line with the one-health approach. Identifying their infectious agents helps to understand existing and emerging diseases. *Escherichia coli* infection, one of the most common bacterial diseases of poultry, causes significant economic losses, and its increasing antibiotic resistance highlights the need for drug susceptibility testing. In this study, the identification and investigation of antibiotic resistance of *E. coli* strains, especially *E. coli* O157, in 33 raptors that act as carriers of this pathogenic bacterium were investigated. For this purpose, 66 swab samples were collected: 33 from the choanal cleft and 33 from the cloaca. Through culture, biochemical testing, and PCR, 48 *E. coli* isolates, including 16 *E. coli* O157 isolates, were identified. *Escherichia coli* isolates were then evaluated for susceptibility to six different antibiotics. Among the 48 *E. coli*-positive samples, the greatest resistance was observed to colistin and neomycin, with sulfamethoxazole and trimethoprim, enrofloxacin, Linco-Spectin (lincomycin hydrochloride and spectinomycin sulfate tetrahydrate), and chloramphenicol showing lesser but still moderate resistance. Given the isolation of *E. coli* (O157) strains from raptors in Kerman province and their observed antibiotic resistance, treatment options are significantly limited. Given the raptors' ability to migrate and travel long distances, these resistant and pathogenic bacteria can be transmitted to humans and domestic animals. Therefore, based on the one-health approach, monitoring, studying, and treating infected raptors in captive environments are particularly important for controlling *E. coli* disease in the context of interactions among animals, humans, and the ecosystem. Further research across broader areas of Iran is essential to identify and manage this disease.

**Keywords:** Raptor, *E. coli* O157, PCR, Antimicrobial resistance (AMR), Kerman province

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## 1 Introduction

Raptors play an important role in their habitat ecosystems due to their location at the top of the food chain (Ford, 2010). These birds can use their flight ability to contaminate the environment with their feces and play an important role in transmitting pathogens to poultry, livestock farms, and groundwater aquifers that supply human water (Gargiulo et al., 2018). Identifying these infectious agents in raptors enables us to prepare to address known and emerging infectious agents (Blomqvist et al., 2012).

The genus *Escherichia* belongs to the *Enterobacteriaceae* family. These gram-negative, rod-shaped bacteria can grow aerobically or anaerobically (Ewing & Edwards, 1986). *Escherichia coli* is a species of *Escherichia* found in the normal intestinal flora of healthy birds, and most of the diseases associated with it occur secondary to environmental stresses in birds (Dho-Moulin & Fairbrother, 1999). The disease can be zoonotic and transmitted through feces contaminated with food or directly to humans (Callaway et al., 2014). *Escherichia coli* O157 and other Shiga toxin-producing *E. coli* subtypes can cause a wide spectrum of disease, from mild diarrhea without bleeding to severe diarrhea with bleeding (hemorrhagic colitis) to hemolytic uremic syndrome in humans (Rangel et al., 2005). In a study by Callaway et al. (2014) of 309 faecal samples from migratory brown-headed cowbirds, 51 common grackle, and 16 bitterns during the migration season in Texas, 11 (3.6%) of migratory brown-headed cowbirds and 3 (5.9%) of common grackle were found to be infected with *E. coli* O157 (Callaway et al., 2014). Clinical signs can range from asymptomatic cases to unresponsiveness immediately before death, depending on the specific *E. coli* disease. Localized infections usually show fewer and milder clinical signs than systemic infections. Birds that develop colisepticemia often become very lethargic or die in the final stages.

Reduced water intake is a sign of a decreasing chance of recovery. When a severely infected bird is approached, they are often unresponsive to stimulation and easily handled. These birds usually sit in a hunched position with their eyes closed and their head, neck, and wings drooping. Their beaks may be placed on the ground to support their heads. Dehydration is characterized by dry, dark skin, especially on the legs and feet. Dehydrated young chicks will clearly have

raised folds in the mid-section and around the skin of the legs. Birds that develop colisepticemia often become very lethargic or die in the final stages (Immunology & Disease, 2010; McPeake et al., 2005; Radhouani et al., 2012; Stromberg et al., 2017).

Antimicrobial resistance (AMR) in bacterial cultures from a variety of human, animal, food, and environmental sources is increasing daily, indicating the need for drug susceptibility testing to identify resistant isolates in laboratories. Specifically, AMR has been steadily increasing among *E. coli* isolates, and drug resistance genes have been transferred to *E. coli* isolates (Pormohammad et al., 2019). Given that raptors are exposed to antibiotics through various routes, such as sewage, hospitals, and household waste, they can be used to screen for antibiotic resistance genes and multidrug-resistant bacteria (Jurado-Tarifa et al., 2016). Any role that wildlife may play in the transfer of antibiotic-resistant bacteria between humans and the environment is of concern and should be investigated. Considering the epidemiological significance of birds in *E. coli* infections, the zoonotic nature of this pathogen, the relevance of the One Health approach, and the absence of prior research on this disease in Iranian raptors, this study aimed to investigate the presence of *E. coli*, isolate the O157 serotype, and assess the antibiotic resistance of all identified *E. coli* isolates from raptors admitted to the quarantine facility at Kerman's Department of Environment.

## 2 Material and Methods

**Sampling:** During this study, which lasted from April to October 2023, 33 swabs were collected from the choanal cleft, and 33 swabs from the cloaca of live raptors submitted for quarantine at the Kerman Environmental Protection Department (Table 1). Most of the birds had a body condition score of 2-3, were fed chicken meat or an unknown diet, and were raised in captivity. In terms of clinical signs, they showed these symptoms: diarrhea, anorexia, lethargy, and ruffled feathers. After restraining the raptors, autoclaved swabs moistened with distilled water were used to sample the choanal cleft and cloaca of all 33 raptors. These swabs were placed in tubes containing Kerry Blair transport medium (Merck, Germany) and transported on ice to the microbiology laboratory of the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman.

**Table 1.** Information on birds of prey referred to the Kerman Province Environmental Department

Species	Number of birds examined	Temperature range	Type of housing	Age	Body condition score
Common kestrel ( <i>Falco tinnunculus</i> )	17	30°C-36°C	quarantine	mature	Mostly 2-3
Peregrine falcon ( <i>Falco peregrinus</i> )	2	30°C-36°C	quarantine	mature	Mostly 2-3
Long-legged buzzard ( <i>Buteo rufinus</i> )	10	30°C-36°C	quarantine	mature	Mostly 2-3
Eurasian sparrowhawk ( <i>Accipiter nisus</i> )	1	30°C-36°C	quarantine	mature	
Black kite ( <i>Milvus migrans</i> )	1	30°C-36°C	quarantine	mature	
Northern goshawk ( <i>Accipiter gentilis</i> )	1	30°C-36°C	quarantine	mature	
Cooper's hawk ( <i>Accipiter cooperii</i> )	1	30°C-36°C	quarantine	mature	

Isolation of *E. coli*: Once the microbiology laboratory received samples, the swabs were placed in Peptone Water Broth (Merck, Germany) and incubated at 37°C (98.6°F) for 24 hours. The swabs were then cultured using the streak plate method on MacConkey agar (Merck, Germany) and incubated at 37°C (98.6°F). After 24 hours, the plates were removed from the incubator and examined. Bacteria that had pink, smooth, convex, round, and medium-sized colonies on MacConkey agar plates were selected as suspected *E. coli* colonies. From each sample culture, two single pink colonies (lactose positive) were selected as suspected *E. coli* isolates for confirmatory biochemical tests, including Gram staining, Triple Sugar Iron agar, Methyl Red and Voges-Proskauer, sulfur, indole, motility (SIM), and citrate media (Markey et al., 2013; Washington, 1985).

DNA extraction and PCR to detect the specific gene of *E. coli* O157: One to two colonies were taken and suspended in a 0.5 mL sterile microtube, containing 350 µL of distilled water. The microtubes were heated (dry bath or heating

block) for 10 to 15 minutes at 100 °C (212°F). Then, the microtubes were cooled for 5 minutes at -20 °C (-4°F), and the samples were centrifuged at 10,000 g for 60 seconds. Finally, 200 µL of the supernatant was removed, transferred to a 1.5 mL sterile microtube, and frozen at -18 °C (-0.4°F) for use in the next steps. The PCR reaction was performed in a final volume of 25 µL, including 12 µL of PCR Master Mix Red, seven µL of sterile distilled water, 0.5 µL of Forward primer, 0.5 µL of Reverse primer, and five µL of extracted DNA (Table 2). The thermal program was performed according to the protocol described in Table 3 (Paton & Paton, 1998). To identify positive strains for the genes and sequences of interest, all PCR products were electrophoresed on a 2% agarose gel at 100 V for 40 min. To examine the PCR results, images of the bands were photographed using a GelDoc 1000 imaging system (Vilber Lourmat, Collégien, France).

**Table 2.** Primer sequence used (Paton & Paton, 1998)

Bacteria	Primer	Sequence (5'-3')	Amplicon size (bp)
<i>Escherichia coli</i> O157	<i>rfbO157</i>	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259

**Table 3.** Thermal program for PCR products of O157

Cycles	Denaturation	Annealing	Extension
1-10	95 °C for 1 min	65 °C for 2 min	72 °C for 1 min
11	95 °C for 1 min	64 °C for 2 min	72 °C for 1 min
12	95 °C for 1 min	63 °C for 2 min	72 °C for 1 min
13	95 °C for 1 min	62 °C for 2 min	72 °C for 1 min
14	95 °C for 1 min	61 °C for 2 min	72 °C for 1 min
15-24	95 °C for 1 min	60 °C for 2 min	72 °C for 1.5 min
25-35	95 °C for 1 min	60 °C for 2 min	72 °C for 2.5 min

Antibiotic susceptibility testing: In this study, antibiotic susceptibility testing was performed using six antimicrobials (colistin (10 µg), sulfamethoxazole and trimethoprim, enrofloxacin (5 µg), lincomycin, chloramphenicol (30 µg), and neomycin (30 µg)) (Padtan Teb Company, Iran).

Initially, the target bacteria were cultured in nutrient broth (Merck, Germany). After the bacteria grew to 0.5 McFarland standard ( $1.5 \times 10^8$  CFU/mL), a sample was taken from the nutrient broth using a sterile swab and cultured on Mueller-Hinton Agar (Merck, Germany) using the lawn culture

method. Finally, the diameter of the zone of bacterial non-growth around the antimicrobial discs was measured using a caliper. The results obtained were compared with the standards presented in CLSI (2018) (Lubbers et al., 2018; M, 2024; Pierce & Mathers, 2022).

### 3 Results

In this study, of the 66 swabs taken, 61 swabs formed single, round, pink to red, medium-sized colonies with a convex surface on MacConkey agar that were capable of

lactose fermentation, suspected to be *E. coli*. No growth was observed from 5 swabs. From these colonies, isolates were taken for confirmation by biochemical tests. Forty-eight samples identified as indole producers in biochemical tests, without hydrogen sulfide production, MR-positive, VP-negative, and lacking citrate, were confirmed as *E. coli*. As a result of the findings from the culture and biochemical tests, 48 isolates of *E. coli* were isolated from 29 birds, 27 were from cloacal samples, while 21 were from the choanal cleft (Table 4 and Table 5).

**Table 4.** Number of positive biochemical and PCR test samples

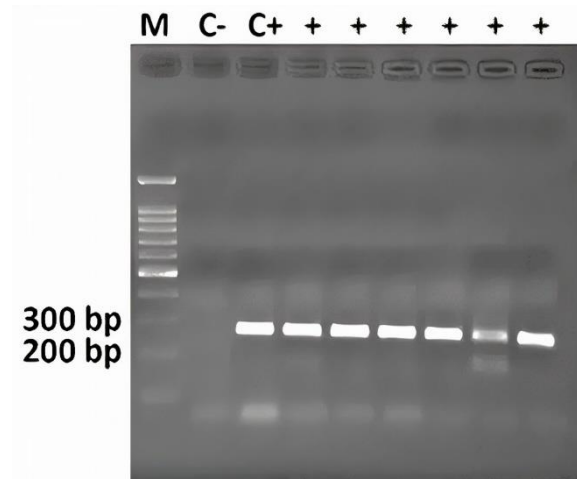
	Total samples	Choanal Cleft sample	Cloacal sample
Total number	66	33	33
Positive <i>E. coli</i> with biochemical tests	48	21	27
<i>E. coli</i> O157 positive by PCR	16	8	8

**Table 5.** Isolation of *E. coli* from birds of prey referred to the Kerman Province Environmental Department

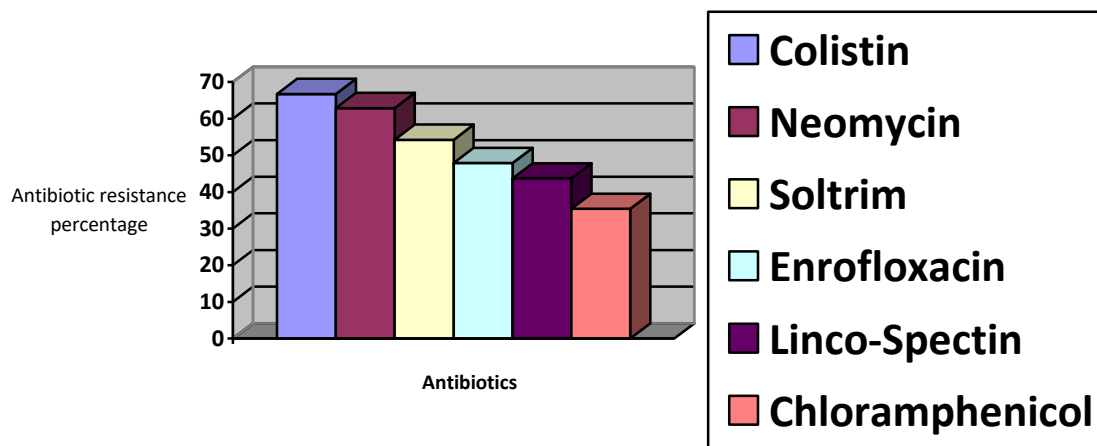
Species	Number of birds examined	Number of positive <i>Escherichia coli</i>	Isolation percentage	Number of positive <i>E. coli</i> O157
Common kestrel ( <i>Falco tinnunculus</i> )	17	15	88	7
Peregrine falcon ( <i>Falco peregrinus</i> )	2	2	100	0
Long-legged buzzard ( <i>Buteo rufinus</i> )	10	9	90	4
Eurasian sparrowhawk ( <i>Accipiter nisus</i> )	1	1	100	1
Black kite ( <i>Milvus migrans</i> )	1	1	100	0
Northern goshawk ( <i>Accipiter gentilis</i> )	1	1	100	1
Cooper's hawk ( <i>Accipiter cooperii</i> )	1	1	100	0

PCR identified 16 *E. coli* O157 in 16 samples from 13 birds. Eight cloacal samples and eight choanal cleft samples were positive for *E. coli* O157 (Figure 1).

In this study, the results show that among 48 *E. coli* isolates, the highest antibiotic resistance was observed to colistin, neomycin, sulfamethoxazole and trimethoprim, enrofloxacin, Lincomycin, and chloramphenicol, respectively (Figure 2).



**Figure 1.** Electrophoresis of PCR products to detect the specific gene of *E. coli* O157 positive (bp259): bp100 marker (M), negative control (C-), positive control (C+), negative sample (-), positive sample (+).



**Figure 2.** Comparison chart of antibiotic resistance frequencies in *E. coli* isolates

#### 4 Discussion

A one-health approach, linking wildlife, the environment, and human health, is essential for understanding and managing the health risks associated with *E. coli* in raptors and their potential impacts on ecosystems and human health. The isolation of *E. coli* species in 48 of 66 samples (72.2%) indicates a significant level of exposure or infection with this disease in the raptor population of Kerman province. Foodborne *E. coli* are largely resistant to common antimicrobials and can colonize and cause disease in humans; in addition, these bacteria have the potential to transfer antimicrobial resistance genes to other *E. coli* species present in the human gastrointestinal tract (Hammerum & Heuer, 2009). This level of contamination

could pose a threat to the health of wildlife, other animals, and humans through environmental exposure. This threat is further exacerbated by the migration and movement of raptors in this region; the presence of this factor could play a role in the spread of the bacteria to humans, other animals, and the environment. Various studies have investigated the prevalence of *E. coli* in wild birds; for example, in a study conducted on 145 dead and euthanized wild birds in Naples, Italy, by Gargiulo et al. (2017), 6.8% of the samples were carriers of *E. coli*, including 3 *E. coli* O26, 2 *E. coli* O55, and 5 *E. coli* O145 (Gargiulo et al., 2018). In another study by Bertelloni et al. (2019) on the intestines of 121 wild birds in central Italy, 17.3% of the samples contained at least one virulence gene of *E. coli*, including *eaeA*, *hlyA*, *stx1*, and *stx2* (Bertelloni et al., 2019). In a study by Mousavinezhad et al.



(2024) on 184 wild passerines in northeastern Iran, 70.1% of cloacal swab samples contained *E. coli*, and 79.6% of these isolates carried at least one virulence gene: *hlyA*, *fimH*, *AFA*, *cnf1*, *aer*, and *papC* (Mousavinezhad et al., 2024). In a study conducted by Nandhini et al. (2024) in the Nilgiris, India, 45% of samples prepared from feces, cloacal swabs, and footpads of 50 pigeons were contaminated with potentially pathogenic *E. coli*, which showed varying degrees of antibiotic resistance (Nandhini et al., 2024). In the study by Sigirici et al. (2020), 37.7% of cloacal swab samples isolated from 116 parakeets, 56 canaries, 56 parrots, and four finches in Istanbul veterinary centers were contaminated with potentially pathogenic *E. coli* (Diren Sigirci et al., 2020). In the present study, 72.7% of *E. coli* were isolated from choanal cleft and cloacal samples, and these samples require further investigation to confirm their pathogenicity using virulence genes. The prevalence of pathogenic *E. coli* can depend on various factors, including virulence factors, host susceptibility, host health, predisposing and immunosuppressive factors such as toxins, nutritional deficiencies, viral infections, and stressors. These factors may explain the variability in the reported percentage of pathogenic *E. coli* involvement across studies. The source of bacteria in raptors is their prey, such as rodents and pigeons (Nolan et al., 2017).

On the other hand, raptors become reservoirs of this bacteria by hunting these animals. Raptors can cause contamination of industrial poultry in various ways; for example, consumption of water from wells contaminated by feces of raptors in poultry farms causes the transmission of these bacteria to industrial poultry, which in turn can cause conflict between humans and raptors in captivity through consumption of meat from these industrial poultry (Nolan et al., 2017). Due to the proximity of the habitat of the common kestrel and the long-legged buzzard to cities, the probability of their feeding on rodents and pigeons increases. This may also explain the higher percentage of conflicts between the common kestrel and the long-legged buzzard compared to other species in the present study.

*E. coli* disease in birds can range from asymptomatic to sudden death. Clinical signs of the disease include lethargy, weight loss, loss of appetite, ruffled feathers, diarrhea, darkening of the skin, especially the legs, and death. Among these symptoms, lethargy, loss of appetite, ruffled feathers, and diarrhea were observed in most of the birds studied, which could be associated with the isolated *E. coli* (7–9).

In this study, the identification of *E. coli* O157 in 16 samples (24.2%) is also of great importance, as it could be

one of the most pathogenic serotypes among the species associated with *E. coli* disease in humans and animals. In a study conducted by Mousavi et al. (2020) involving 500 chicken meat samples collected from Isfahan poultry farms, it was discovered that 44 of these samples (8.8%) contained *E. coli* O157.(25). In a study by Callaway et al. (2014) on 309 fecal samples from migratory brown-headed cowbirds, 51 common grackles, and 16 bitterns during the migration season in Texas, it was found that 11 (3.6%) of migratory brown-headed cowbirds and 3 (5.9%) of common grackles were infected with *E. coli* O157 (5). In this study, since the majority of the raptors in Kerman's Department of Environment were fed chicken meat, this is suspected to be the reason why these birds were contaminated with *E. coli* O157.

Antimicrobial resistance is relatively common in bacteria isolated from domestic poultry, but has also been reported in bacteria isolated from wild birds. In a study by Guenther et al. (2010) of 15 common European wild bird families, including the common kestrel and the Eurasian goshawk, four families (26.6%) were resistant to chloramphenicol, two families (13.3%) were resistant to gentamicin, five families (33.3%) were resistant to neomycin, and two families (13.3%) were resistant to fluoroquinolones (Guenther et al., 2010). In a study by Vredenburg et al. (2013), 76 *E. coli* isolates isolated from the feces of raptors in Spain and Sweden were evaluated for antimicrobial resistance, and 51 (67.1%) were found to be resistant to sulfamethoxazole and trimethoprim, and all isolates were susceptible to gentamicin (an aminoglycoside) (Vredenburg et al., 2014). In a study of feces samples from 20 birds of prey, including the common buzzard, eurasian goshawk, and the peregrine falcon in southern Italy by Varriale et al. (2020), two (13.3%) were resistant to sulfamethoxazole and trimethoprim and two (13.3%) were resistant to gentamicin, and none of the isolates were resistant to enrofloxacin (Varriale et al., 2020). In a study by Şahan Yapıcıer et al. (2022) on fecal and intestinal samples of 82 wild birds in a rehabilitation center in Turkey, a total of 51 *E. coli* strains were isolated from 22 different wild bird species, including the long-legged buzzard, common kestrel, and Eurasian goshawk. The antimicrobial resistance of these 22 species was as follows: resistance to enrofloxacin was observed in 11 isolates (21.6%), resistance to amphenicols (florfenicol) in 21 isolates (41.2%), resistance to sulfamethoxazole and trimethoprim in 25 isolates (49%), resistance to neomycin in 11 isolates (21.6%), and resistance to ciprofloxacin in 11 isolates (29.4%) (Şahan Yapıcıer et al., 2022). In a study by

Jackson et al. (2023) on samples taken from the cloaca of 50 wild birds in Molongo, Brazil, which included 28 different species, it was found that 13 of them (26%) had *E. coli* bacteria, of which one (7.7%) was resistant to gentamicin, two (15.4%) to azithromycin, one (7.7%) to tobramycin, seven (53.8%) to ampicillin, two (15.4%) to ceftriaxone, two (15.4%) to Co-amoxiclav, one (7.7%) to tetracycline, two (15.4%) to sulfonamide, two (15.4%) to nalidixic acid, and one (7.7%) to meropenem. All *E. coli* samples were susceptible to ciprofloxacin (Jackson et al., 2021). In general, the lowest antibiotic resistance of *E. coli* isolated in the studies reviewed was to the amphenicol family, and the highest resistance was to the antibiotics sulfamethoxazole and trimethoprim, followed by the aminoglycoside family. In the present study, the lowest resistance to the amphenicol family and the highest resistance among the mentioned antibiotics were observed in the aminoglycoside family, followed by Soltrim.

Previous studies have established high rates of antibiotic resistance in *E. coli* from domestic poultry. These studies consistently show the highest resistance to sulfamethoxazole and trimethoprim, and to aminoglycosides like neomycin, and the lowest resistance to Lincomycin and the amphenicol family. (Azizpour, 2022; Hardiati et al., 2021; Salehi & Ghanbarpour, 2010; Sepehri, 2006)

In contrast, the present study found the highest resistance in raptors to polymyxins and aminoglycosides, and the lowest to amphenicols and Linco-Spectin. This similarity in resistance profiles suggests that raptors may acquire antibiotic-resistant *E. coli* through preying on domestic birds.

Future research should focus on analyzing the virulence genes of isolated strains and on expanding sampling to broader geographic areas to better understand the prevalence and impact of this bacterium across different raptor species.

## 5 Conclusion

Given the isolation of *E. coli* (O157) from raptors in Kerman province and the observed antibiotic resistance, treatment options are significantly limited. Given the ability of raptors to migrate and move for long periods of time, they can transmit these resistant and pathogenic bacteria to humans and domestic animals; therefore, based on a one-health approach, monitoring, studying, and treating infected raptors in captive environments is of particular importance for the control of infections caused by *E. coli* in the interaction between animals, humans and the ecosystem.

Further research across broader areas of Iran is essential to identify and manage this disease.

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## Conflict of Interest

We declare that no conflict of interest.

## Author Contributions

H.Sh.: writing-original draft, conceptualization, methodology; M.J., H.T., S.A.: methodology; M.Z.: methodology and writing-original draft.

## Data Availability Statement

Data are available from the corresponding author upon reasonable request.

## Ethical Considerations

This study was conducted in accordance with relevant ethical guidelines for research involving animals and wildlife. The research protocol was reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Kerman, Iran (Approval Code: 38721060-1402).

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